Adduction of DNA with MTBE and TBA in Mice Studied by Accelerator Mass Spectrometry

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ABSTRACT: Methyl *tert*-butyl ether (MTBE) is a currently worldwide used octane enhancer substituting for lead alkyls and gasoline oxygenate. Our previous study using doubly ^{14}C-labeled MTBE [$(CH_3)_3{}^{14}CO{}^{14}CH_3$] has shown that MTBE binds DNA to form DNA adducts at low dose levels in mice. To elucidate the mechanism of the binding reaction, in this study, the DNA adducts with singly ^{14}C-labeled MTBE, which was synthesized from ^{14}C-methanol and *tert*-butyl alcohol (TBA), or ^{14}C-labeled TBA in mice have been measured by ultra sensitive accelerator mass spectrometry. The results show that the methyl group of MTBE and *tert*-butyl alcohol definitely form adducts with DNA in mouse liver, lung, and kidney. The methyl group of MTBE is the predominant binding part in liver, while the methyl group and the *tert*-butyl group give comparable contributions to the adduct formation in lung and kidney. © 2007 Wiley Periodicals, Inc. Environ Toxicol 22: 630–635, 2007.

Keywords: methyl *tert*-butyl ether (MTBE); *tert*-butyl alcohol (TBA); DNA adduct; accelerator mass spectrometry (AMS)

INTRODUCTION

Methyl *tert*-butyl ether (MTBE) is a worldwide used lead-free antiknock additive increasing the octane number, and a gasoline oxygenate enhancing combustion efficiency to reduce the vehicle exhaust emission of carbon monoxide and hydrocarbons (Taniguchi and Johnson, 1979). As the high content of MTBE is added in gasoline and the MTBE production largely increases, the safety of human exposure to MTBE has become a notable issue, because MTBE is a water miscible and very volatile compound, and it biodegrades slowly in groundwater (Squillace et al., 1997). Human exposure to MTBE is mainly by inhalation of fumes while fueling automobiles, and also through drinking water and dermal contact with MTBE contaminated water (Ahmed, 2001; ACFA and EFOA, 2002).

In rodents and humans, MTBE metabolized to *tert*-butyl alcohol (TBA) and formaldehyde via oxidative demethylation by liver microsomes (Hong et al., 1997; Turini et al., 1998). Formaldehyde is metabolized to formic acid and TBA is further oxidized into 2-methyl-1,2-propanediol and 2-hydroxyisobutyric acid (Nihlén et al., 1999; Dekant et al., 2001). These metabolites are potential binding reagents with DNA to

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form adducts. Tang et al. (1997) found that both MTBE and TBA could cause DNA damage in human leukemia cell in a dose-dependent pattern, while formaldehyde also showed obvious cellular toxic effects. The contribution of formaldehyde to MTBE carcinogenesis was investigated and the results were equivocal. Mackerer et al. (1996) reported that formaldehyde derived from MTBE was responsible for MTBE mutagenicity in an activated mouse lymphoma assay. But, Casanova and Heck (1997) and Hong et al. (1997) concluded that metabolized formaldehyde was not considered responsible for MTBE carcinogenicity in their experiments. The formation of DNA adducts are widely recognized as an early detectable and critical step in the process of chemical carcinogenesis (Poirier, 1997; Garner, 1998). Therefore, direct investigation on DNA adducts is a useful method in assessing the role of formaldehyde and TBA in carcinogenesis of MTBE.

Accelerator mass spectrometry (AMS), an ultrasensitive analytical method for DNA adducts measurement, has an extremely low detection limit, a few DNA adducts per 10^{12} nucleotides (Turteltaub et al., 1990; Kenneth and Karen, 1998). The very high sensitivity of AMS enables it possible to examine a variety of chemical xenobiotics, mostly the small toxic compounds, at a low environmental exposure dose (Garner, 2000).

Our previous work shows that MTBE bound with DNA in mice at low dose level using doubly ^{14}C-labeled MTBE (Du et al., 2005). In this paper, the DNA adducts formed with singly ^{14}C-labeled MTBE or TBA in lung, liver, and kidney in mice were measured by AMS. The results were analyzed by comparing with the previous data obtained by using doubly ^{14}C-labeled MTBE to reveal the formation mechanism of MTBE-DNA adducts.

MATERIALS AND METHODS

Chemicals

^{14}C-Methanol with specific activity of 1 Ci/mol and purity of 96%was purchased from Moravek Biochemicals (Lot No. 123-177-001, Brea, CA). ^{14}C-*tert*-Butyl alcohol [$(CH_3)_3$-^{14}COH] with specific activity of 50.0 Ci/mol and purity of 98% was obtained from American Radiolabeled Chemicals (Lot No. 030711, St. Louis, MO). Methyl *tert*-butyl ether (MTBE; purity 99.8%) was obtained from Aldrich (Munich, Germany). RNase A (Type I-A, from Bovine Pancreas 81 kunits/mg protein 90% RNase A) was purchased from Sigma Chemical (St. Louis). Proteinase K (40 U/mg) was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade or better.

Synthesis and Purification of ^{14}C-Labeled MTBE [$(CH_3)_3CO^{14}CH_3$]

The synthesis and purification of ^{14}C-MTBE followed Duan's method (Duan, 2000) with some modifications to realize the radioactive micro synthesis. Briefly, 1.4 mL 20% (v/v) H_2SO_4, 0.5 mL methanol (12.36 mmol, containing191 μCi ^{14}C-methanol), 0.5 mL TBA (5.26 mmol) were added to a flask. The flask was kept at 85°C in an oil bath refluxing for 40 min. The mixture in the flask was fractionated and the distillate (35–37°C) was collected in a receiver flask, which was kept in ice-salt bath (−18 to −20°C). The side arm of the receiver was introduced into cold water to decrease the release of MTBE. Flask head, water condenser, and distillation takeoff adapter were welded together for the same reason. The total distillation process took about half an hour.

The raw product (0.3 mL) mixed carefully with 200 μL 10% (w/v) Na_2SO_3 and then kept at 4°C for 1 day with shaking gently several times. Then, Na_2SO_3 solution in the lower layer was sucked out and the raw product was washed with an equal amount of fresh Na_2SO_3 solution with the same procedure. Approximately, 0.1 mL final product was obtained after washing four times. No further treatment was performed before the animal studies.

The reliability and reproducibility of the radioactive synthesis were ensured by five times rehearsal of nonradioactive cold experiments. Nuclear magnetic resonance (NMR, Varian Gemini 2000), gas chromatography (GC, Varian, GC-3800, FID detector), and micro IR were used to characterize the product and test the purity of MTBE produced in these cold experiments. ^{1}H NMR spectra were measured in d_6-acetone solution.

Animal Treatment

All the animal studies were carried out in compliance with the regulations of the Local Ethics Committee.

Male Kunming mice (about 25 g) were obtained from Beijing Vitalriver Experimental Animal Technology Co. and randomized into six treatment groups (eight mice per group). Animals were housed in polycarbonate cages with wooden chips bedding and received food and water *ad libitum*. Mice were administered MTBE/TBA by gavage (stomach intubation), using a dosing volume of 8 mL/kg body weight (b.w.). One group of mice exposed to saline solution alone via gavage was used as the control group. Six hours postadministration, mice were sacrificed and organs (liver, kidney, and lung) were collected for DNA isolation. The same organs from every two mice were merged into one sample.

For ^{14}C-MTBE, the administered dose was 1.86, 1.39×10, 1.33×10^2, 9.90×10^2, 1.19×10^4 μg MTBE/kg b.w., respectively, which was prepared by mixing the ^{14}C-MTBE (specific activity 15.45 mCi/mol) with proper amount of nonradioactive MTBE carrier.

For ^{14}C-TBA, mice were administered a dose of 9.90×10^{-2}, 9.90×10^{-1}, 1.00×10, 1.01×10^2, and 9.97×10^2 μg TBA/kg b.w. with specific activity of 1.60, 1.81×10^{-1},

6.88×10^{-2}, 1.38×10^{-2}, and 9.78×10^{-3} mCi/mol, respectively.

All the above dosing solutions were prepared several hours before animal experiments and the radioactivities were determined using a liquid scintillation counter (Packard Tri-cab 2750 TR/LL, Packard Instrument, Meriden, CT). On account of the loss of MTBE due to its high volatility, a mean value of the two measurements, before and just after gavage, was taken as the final measured concentration of MTBE solution.

Isolation of DNA

DNA was extracted from liver, kidney, and lung samples using the method of Gupta (1984). The purity of the extracted DNA was monitored by UV $A_{260\ nm}/A_{280\ nm}$ ratio. Only samples with an absorbance ratio of 1.80 ± 0.05 were used. The isolated DNA was dried in vacuum.

AMS Sample Preparation and Measurement

DNA samples were converted to graphite following Vogel's protocol (Vogel, 1992). Typically, DNA samples, sealed under vacuum in a quartz tube, were oxidized to CO_2 in the presence of CuO. The CO_2 was then cryogenically transferred into a glass tube and reduced to filamentous graphite in the presence of cobalt powder, Zn and TiH_2 in a vacuum conversion system. The ^{14}C contents of MTBE-DNA adducts samples were measured by a 2×0.6 MV Tandem AMS facility (National Electrostatic Co., USA) at the Institute of Heavy Ion Physics, Peking University. The TBA-DNA adduct samples were measured by Shanghai Mini-Cyclotron AMS at Shanghai Institute of Applied Physics, Chinese Academy of Science. AMS measurement gives the $^{14}C/^{12}C$ ratio of the sample with the unit of modern carbon (MC). The ratio could be converted to the number of adducts based on some fundamental data (1 MC equals to 5.9×10^{10} ^{14}C/g of carbon; C content in DNA = 30%; 1 μg DNA = 3240 pmol of nucleotides; $T_{1/2}$ of ^{14}C = 5730 a). Graphite standards prepared from Chinese glucose (Chinese standard, 1.362 MC) were used as a monitor of any ^{14}C cross-contamination. The internal graphite standard, oxalic acid-1 (No. SRM-4990, U.S. National Bureau of Standards), was used for normalizing the $^{14}C/^{12}C$ ratios in the AMS measurements.

RESULTS AND DISCUSSION

Synthesis of ^{14}C-MTBE

Because of the high volatility, the MTBE distillate in the fractionation was collected in a range of 35–37°C, which was far below the normal boiling point (55°C) of MTBE. The lower collection temperature enhanced the purity of the product by depressing the vapor pressure of impurities methanol and TBA. Because treated with Na_2SO_3 solution, the final product contained some inorganic impurities such as water and salt, but they did not influence the AMS analysis of ^{14}C-labeled MTBE. Therefore, no further purification process was needed. The nature of purified MTBE obtained in the cold experiments was confirmed by ^1H NMR spectra (Varian Gemini2000, d_6-acetone, d_{ppm} 3.13 (s, 3H, O—CH_3); d_{ppm} 1.16 (s, 9H, —$C(CH_3)_3$)). IR spectra of purified MTBE and pure MTBE reagent were identical. GC data shows that the purity of final MTBE product is more than 95% by normalizing the integration areas of the chromatographic peaks. The main impurities were TBA and isobutylene, as demonstrated by GC-MS analysis (TBA, mass 0.2%; isobutylene, mass 3.3%; MTBE, mass 96.4%). The overall chemical yield of MTBE from TBA was about 20% and the conversion of ^{14}C-methanol was 8%. No detectable methanol and its derivatives were found in the product. The radiochemical purity of ^{14}C-MTBE is very high (>99%) and the specific activity is 15.45 mCi/mol.

Formation of MTBE-DNA Adducts

Figures 1 and 2 show the dose–response relationship of ^{14}C-MTBE binding to DNA in mouse liver, kidney, and lung. The liver DNA adducts increase with increasing dose in a good log/log linear mode over the entire range of the dosage (Fig. 1). In Figure 2(a,b), MTBE-DNA adducts formed in the kidney and lung are only detectable at the dose above 1.33×10^2 μg MTBE/kg b.w. The data demonstrate that MTBE does bind with DNA in the mouse liver, kidney, and lung via its metabolites of the methyl group. The order of adduct concentrations in different tissues was liver > lung > kidney, indicating that the target organ of the methyl group is liver. This result is quite different from

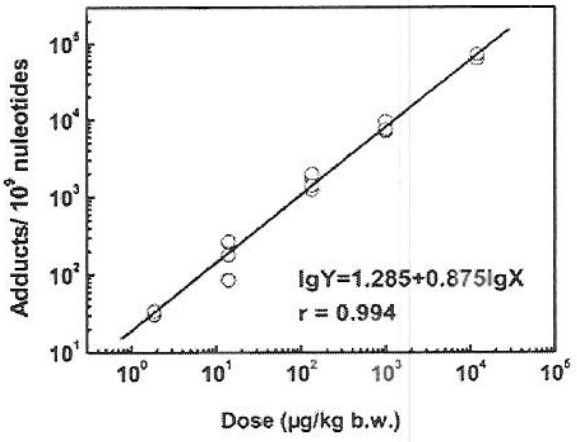

Fig. 1. Dose–response of ^{14}C-MTBE binding to DNA in the mouse liver at 6 h postadministration (two to four parallel samples).

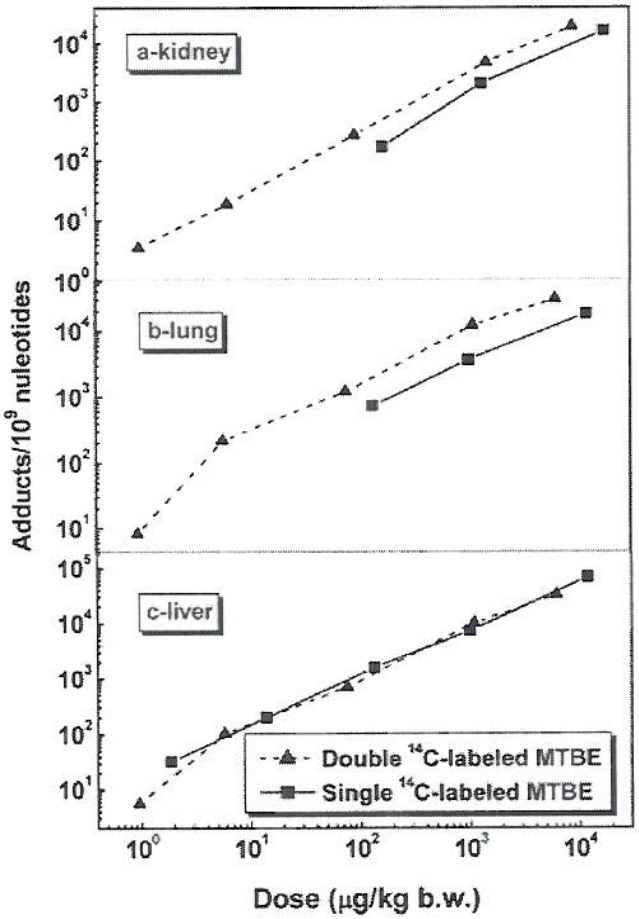

Fig. 2. The DNA adduction comparison of doubly ^{14}C-labeled MTBE (Du et al., 2005) and singly ^{14}C-labeled MTBE in mouse kidney (a), lung (b), and liver (c). The numerical values in these figures are mean values of two to four parallel samples.

the previous result by using the doubly ^{14}C-labeled MTBE (Du et al., 2005), in which the order was lung > liver > kidney. Interestingly, the order observed here is as same as that in our previous AMS study on the formation of DNA adducts with formic acid (Wang et al., 2004). Figure 2(c) shows that the adduct level of singly ^{14}C-labeled MTBE is comparable to that of doubly ^{14}C-labeled MTBE at the same doses in liver (Du et al., 2005), implying that the methyl group of MTBE is the predominating binding pathway while the *tert*-butyl group only makes little contribution to the formation of DNA adducts in mouse liver. In addition, we notice that the MTBE-DNA adduct level (Du et al., 2005) and the formic acid–DNA adduct level (Wang et al., 2004) observed in mouse liver are very similar. Thus, we speculate that the metabolite formaldehyde obtained from MTBE can be further oxidized to formic acid *in vivo* (Dekant et al., 2001), which plays a pivotal role in forming methylated DNA adducts (Wang et al., 2004), just as in the case of the formation of the formic acid–DNA adducts.

Kryukov et al. (1997) reported the formulation of nucleosides by an esterification between formic acid and 5′-OH group of pentose *in vitro*. Cheng (2004) has repeated this experiment in our laboratory and confirmed the report of Kryukov et al. (1997). Their findings afforded us a clue that a potential way of DNA adduction with MTBE is through the metabolite formic acid by covalently binding to the 5′-O position of pentose, thus resulting in the cleavage of the DNA strand. But, at the same time, incorporation of formic acid into DNA also contributed to the radioactivity of the DNA samples. In the incorporation process, formic acid, via the transfer reaction of tetrahydro-folic acid, becomes the source material in the synthesis of the nitrogen base moieties (A, G, C and T) of DNA. Such incorporation of formic acid into the DNA molecule does not bring about any genotoxicity towards DNA, nor risk of carcinogenesis in the living organism. It is therefore concluded that the adduction pathway is toxic, whereas the incorporation is benign. According to our preliminary experimental results, the majority of formic acid would be incorporated into DNA, but does not form the harmful adducts with DNA.

The results of kidney and lung show great similarity within the whole tested dose range but are quite different

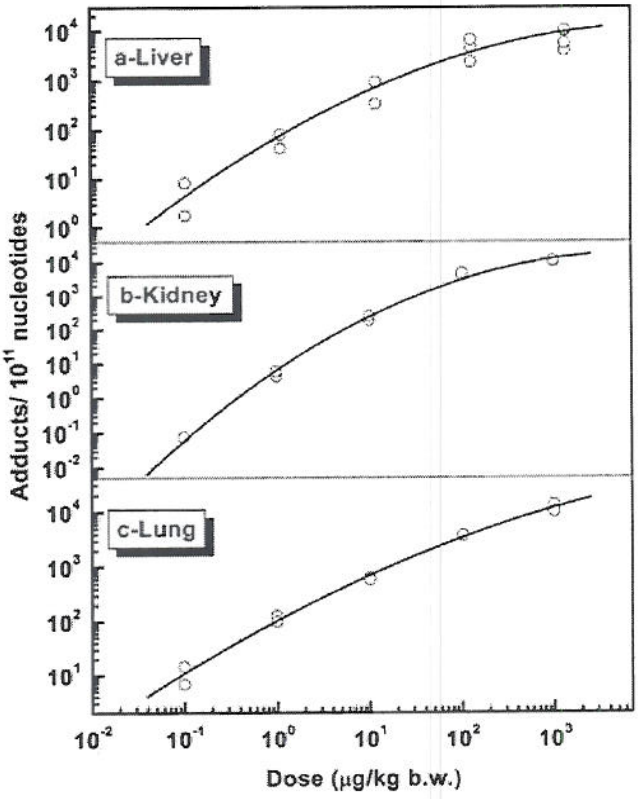

Fig. 3. Dose–response of ^{14}C-*tert*-butyl alcohol binding to DNA in mouse liver, kidney, and lung at 6 h postadministration. (a) Liver (two or three parallel samples), (b) kidney (one or two parallel samples), (c) lung (two parallel samples).

from that of liver [Fig. 2(a,b)]. In the low dose range from 0.36 to 1.39 × 10 μg /kg b.w., adducts are undetected in our experiment. From dose 1.33 × 10^2 μg/kg b.w. to 11.9 mg MTBE/kg b.w., the DNA adducts increase with increasing dose. A large number of DNA adducts observed in the doubly ^{14}C-labeled experiment (Du et al., 2005), but in this study no detectable DNA adducts with radio-methyl labeled MTBE was found at low dose levels. Therefore, we believe that the *tert*-butyl group of MTBE might be the predominant binding pathway in kidney and lung at low dose level, such as 1.4 μg /kg b.w. (Brown, 1997). But at high dose levels, *tert*-butyl group along with its metabolites form adducts in the same order of magnitude as that of the methyl group.

Formation of TBA-DNA Adducts

The TBA-DNA adducts in mouse liver, lung, and kidney were studied by AMS to confirm whether TBA could form DNA adducts *in vivo*. Figure 3 shows TBA-DNA adducts are definitely formed in liver, lung, and kidney with positive dose–response relationship. In mouse liver, MTBE-DNA adduct number by using doubly ^{14}C-labeled MTBE was about two orders of magnitude higher than that of TBA-DNA at the same mol/kg b.w. dosages. This result accords well with the finding that the methyl group of MTBE is the predominant binding pathway in liver. However, in lung and kidney, the *tert*-butyl group also plays an important role in the formation of DNA adducts.

Although the role of DNA adducts in carcinogenesis process is still not fully established, in most cases DNA adduct level is positively correlated with the genotoxicity and hence the carcinogenicity of the chemicals (Otteneder and Lutz, 1999). As the TBA-DNA adducts level is considerably lower than the MTBE-DNA adducts level, TBA seems less genotoxic than MTBE. However, at present we are unable to elucidate the reason of the remarkable difference among the different organs.

CONCLUSION

MTBE labeled with ^{14}C at the methyl group was prepared from ^{14}C-methanol and *tert*-butyl alcohol. The methyl part of MTBE definitely formed adducts with DNA *in vivo* in mouse liver, lung, and kidney and it was a predominant binding pathway in liver. The benign incorporation process of methyl part of MTBE into DNA in large extent was speculated. TBA was found, for the first time, to form DNA adducts in mouse liver, lung, and kidney.

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